931,635

PATENT SPECIFICATION

DRAWINGS ATTACHED

931,635

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COMPLETE SPECIFICATION

Method of producing a Proteolytic Enzyme by use of Black Aspergillus Type Molds

We, NODA INSTITUTE FOR SCIENTIFIC RESEARCH, an incorporated body organised under the laws of Japan, of 339, Noda, Noda-shi, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to a method of producing a proteolytic enzyme by use of black Aspergillus type molds.

The present invention is an improvement of a method of U.S. Patent No. 2,848,371.

Concerning the formation of protease by use of Aspergillus molds, there have heretofore been various researches on proteases having activity in neutral to alkaline ranges. As for the formation of protease having activity and optimum pH at approximately 3.0, and having resistivity to acid, the amount produced is smaller and the distribution of the molds producing the same is narrower.

There has been little systematic investigation until now on the formation of protease having optimum pH approximately at 3.0 and having resistivity to acid, excepting that Gorbach et al have reported the formation of a protease by a cultivation in liquid of Aspergillus niger having optimum pH at approximately 4.9. (See G. Gorbach and O. G. Koch, Arch. für Mikrobiologie *23*, 265 and 284 (1955)).

The present inventors have discovered after systematic investigations of cultivating conditions for the production of the acid-resisting protease by use of black Aspergillus type molds belonging to Kuro-Koji mold group. (See Sakaguchi, Iizuka, and Yamaguchi, J. Applied Mycology (Univ. of Hokkaido), *3*, 54 (1949); Ibid. *3*, 97 (1950); and *4*, 1 (1950); Journal of the Agricultural Chemical Society of Japan *24*, 138 (1951)) that the production of the acid-resisting protease by use of black Aspergillus type molds can be greatly increased, for example by 40 to 90%, compared with the control.

The positions of the molds in the classification of Aspergillus will be understood from the following Table.

(K. Sakaguchi, H. Iizuka and S. Yamazaki: J. Agr. Chem. Soc., Japan, vol. 24, 138, 1951; and H. Iizuka: J. General and Applied Microbiology, vol. 1, No. 1, 10, 1955).

(1) Conidial wall with coloured bars when mature ——A. niger group.

(1) Conidial wall smooth, rough or rarely echinulate ——Kuro-Koji mold group.

(2) Colonies black ——(3)

(2) Colonies with brown or olive shades——(7)

(3) Assimilate nitrites*——(4)

(4) Ist sterigmata over 30μ ——A. batatae, Saito.

(4) Ist sterigmata 13—25μ ——(5)

(5) Yellow pigment produced ——A. usamii nov. sP.

(5) Yellow pigment not produced ——A. usamii var. R—17 nov. var.

(3) Do not assimilate nitrites ——(6)

(6) Ist sterigmata 15—23μ, conidiophore 2—3mm. or more ——A. saitoi nov. sP.

(6) Ist sterigmata up to 16μ, colonies somewhat mummy brown ——A. saitoi var. R—16 nov. var.

(6) Ist sterigmata 10—13μ, conidiophore under 1.5 mm., conidial head crowded ——A. saitoi var. Kagoshima nov. var.

*The assimilation of nitrites has been tested by the use of the media; sucrose, 30 gr.: $NaNO_2$, 1.5 gr.; K_2HPO_4, 1 gr.; KCl, 0.5 gr.: $MgSO_4 . 7H_2O$, 0.05 gr.; $FeSO_4$, 0.05 gr.; in distilled water 1l. The species which assimilate nitrites grow readily in 1—2 days at 30—35° C., while the non-assimilating species show none or only scanty growth in the upper part of the agar slant even after 5—10 days.

[*Price 4s. 6d.*]

(7) Colonies with olive shades, assimilate nitrites ——(8)

(8) Sterigmata mostly in double series but often mixed with single series in the same heads, Ist sterigmata 5μ ——A. inuii nov. sP.

(8) Sterigmata in double series ordinary—— A. inuii var. K—19 nov. var.

(8) Conidial heads scanty——A. inuii var. R—7 nov. var.

(7) Colonies with olive shades, assimilate nitrites——(8)

(9) Assimilate nitrites——A. aureus Nakazawa, A. aureus var. minor Nakazawa et Shimo, A. awamori var. fumeus Nakazawa et Shimo, A. aureus F. sP. R—2.

(9) Do not assimilate nitrites ——(10)

(10) Yellow-orange pigment produced in mycelium, conidial heads not seen in ordinary colonies ——A. nakazawai nov. SP.

(10) Yellow-orange pigment not produced ——A. awamori Nazawa et Shimo. A. awamori var. minimus Nakazawa et Shimo, A. awamori var. piceus Nakazawa et Shimo, A. awamori var. fuscus Nakazawa et Shimo, A. aureus var. acidus Nakazawa et Shimo, A. awamori var. mirinus Nakazawa et Shimo, A. awamori F. sP. R—5, R—9, H—2 and R—1.

According to the present invention the production of an acid-resistant protease having an optimum pH for milk casein digestion of approximately 2.7, by cultivation of a mold of black Aspergillus type, is considerably increased by adding an inorganic nitrogenous source compound to the cultivation medium.

The cultivation is preferably carried out at a temperature of 30° C. for a period of at least 60 hours, and the medium may be solid or liquid, in the latter case the pH of the medium during the cultivation should be kept in the range 2.5 to 6.0.

Suitable molds are aspergillus usamii and Aspergillus Saitoi, for which the C/N ratio of the medium is advantageously below 3.2; *Aspergillus aureus* and *Aspergillus awamori*, for which the C/N ratio of the medium is advantageously approximately 5; and Aspergillus Inuii and *Aspergillus Nakazawai* for which the C/N ratio of the medium is advantageously approximately 8.

Preferred inorganic nitrogen source compounds are NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, $NaNO_3$ and KNO_3, and these are desirably employed the proportion by weight of 0.5% to 5% based on the weight of basal solid medium: the ammonium salts the preferred proportion is 0.25% to 3%, for the inorganic nitrates 0.5% to 3%.

The invention will now be described in greater detail by way of example, with reference to the accompanying drawings.

Strains employed in the following examples are of Kuro-Koji mold group preserved in the Institute of Applied Microbiology, University of Tokyo, of Aspergillus flavasoryzae preserved in the Arima Research Laboratory, University of Tokyo, and of Aspergillus sojae preserved in the Noda Institute for Scientific Research.

Enzymatic solution for solid cultivation is made as follows: Five grams of wheat bran is added to 3.5 ml. of water, and sterilized under pressure in the conventional manner. The bran is then inoculated with a strain of a mold and incubated at 30° C. for about 64 hours. After extraction with 50 ml. of aqueous hydrochloric acid (pH 2.7) and filtration, the filtrate is diluted with N/10 acetate buffer (pH 2.7) up to ten times of the volume to make an enzymatic solution. In the ensuing description the amount of inorganic nitrogen source compound added to the basal culture medium of wheat bran will be expressed as weight % based upon the weight of the wheat bran.

Enzymatic solution for liquid cultivation (submerged) is made as follows: Three grams of a mixture of wheat bran and defatted soybeans are added to 100 ml. of water in a 500 ml.-content shaking flask and sterilized under pressure in the conventional manner. The mixture is inoculated with a strain of a mold and incubated at 30° C. on a shaking machine operated at 140 reciprocations per min. After incubation for a definite period of time, the mixture is filtered, and the filtrate is diluted with N/10 acetate buffer (pH 2.7) up to ten times of the volume to make an enzymatic solution.

The protease assay of the enzymatic solution in the invention is carried out as follows: The enzymatic solution containing the protease is made to act on a substrate of 2% milk casein (Hammarsten) at 30° C. for 10 min., according to the modified Anson's method. After removal of the precipitate formed by trichloroacetic acid by filtration, the filtrate is added to 1 ml. of Folin's reagent and the color which appears is colorimetrically measured at 660 mμ by use of a photometer (Hitachi Ltd., EPO—B type), showing as ΔO.D.

After preliminary experiments, it was found that 50 to 70% of moisture content and cultivation conditions at temperature of 30° C. for 62 to 64 hours are adequate for the solid cultivation. Also, the relationship between the amount of the acid protease formed and the proportion of wheat bran to defatted soybeans was determined in the cultivation in liquid (submerged), for determination of the basal medium for various strains belonging to Kuro-Koji mold group. The results are shown in Figure 1. These experiments show that *Aspergillus Usamii*, *Aspergillus Saitoi* and others prefer nitrogen source in a more concentrated state, *Aspergillus Inuii*, *Aspergillus Nakazawai* prefers nitrogen source in a comparatively lower concentration, and *Aspergillus aureus*, *Aspergillus awamori*, and others prefer nitrogen of the intermediate concentration.

Now, the effect of the addition of various

kind of nitrogenous compounds on formation of the acid protease of black Aspergillus type molds, according to the present invention, will be set forth below.

Figure 2 is a graph showing the effect of the addition of various kind of nitrogeneous compounds on formation of the acid protease by use of *Aspergillus Saitoi* in a solid cultivation. This experiment teaches that addition of various kind of nitrogeneous compounds remarkably increases formation of the acid protease, that the preferable C/N ratio of the medium (ratio of carbon % to nitrogen % in the medium) somewhat varies according to the kind of nitrogenous compound, but it may generally be approximately 8.5, and that inorganic nitrogenous sources, such as nitrates and ammonium salts, are exceedingly effective in promoting the protease formation even when present in comparatively small amounts. Thus, use of an inorganic nitrogenous source seems to be materially advantageous from economical points of view, over use of an expensive organic nitrogenous source.

Outside of the nitrogenous compounds, some of potassium salts, calcium chloride, and others seem to be slightly effective, but various carbon sources and various metallic salts are rather inhibitory to the formation of the protease.

Figure 3 is a graph showing the variation of the amount of the acid protease formed as time elapses, in the cases where 0.5% of sodium nitrate and 1% of ammonium chloride are respectively added.

There have been few reports that teach the efficacy of the addition of inorganic nitrogenous sources in solid cultivation for the formation of the acid protease. Sakamoto et al alone, have found that addition of ammonium sulfate increased the formation of the enzyme in the case of Penicillium, i.e. by 122% based upon the control. (See J. Fermentation Technology *35*, 386 (1957)).

In the following Table 1, typical strains of black Aspergillus type molds belonging to Kuro-Koji mold group are listed, which give remarkably increased acid protease formation by addition of the inorganic nitrogenous source.

Table 1. Typical strains giving increased formation of the acid protease by addition of an inorganic ammonium salts or nitrates.

Aspergillus Usamii	(ATCC No. 14331)
Aspergillus Saitoi	(ATCC No. 14332)
Aspergillus Inuii	(ATCC No. 14333)
Aspergillus aureus	(ATCC No. 14334)
Aspergillus awamori	(ATCC No. 14335)
Aspergillus Nakazawai	(ATCC No. 14336)

Further, the effects of inorganic nitrogen compounds on the formation of various kinds of proteases by use of black Aspergillus molds belonging to *Aspergillus Saitoi*, Aspergillus molds belonging to *Aspergillus oryzae-flavus*, and other Aspergilli, are shown in Table 2. As seen from the table, addition of an inorganic nitrogen source does not increase acid protease in Aspergilli other than black Aspergillus molds, but increases alkaline protease of pH 7.5.

TABLE 2

Effect of Inorganic Nitrogen Compounds in the formation of proteases by various species of Aspergilli

Strain	Final pH*			Acid Protease			Neutral Protease			Alkali Protease		
	Control	1% NH_4Cl	0.5% $NaNO_3$	Control	1% NH_4Cl	0.5% $NaNO_3$	Control	1% NH_4Cl	0.5% $NaNO_3$	Control	1% NH_4Cl	0.5% $NaNO_3$
A. Saitoi	3.8	4.3	3.8	0.600	0.946	0.932	0.008	0.006	0.008	0	0	0
A. oryzae var magnasporus	6.2	6.8	6.7	0.223	0.158	0.127	0.343	0.328	0.517	0.266	0.263	0.450
A. oryzae Wehmer	5.9	6.2	6.2	0.100	0.071	0.077	0.155	0.101	0.168	0.100	0.054	0.130
A. oryzae Wehmer	6.1	6.0	6.2	0.115	0.089	0.097	0.204	0.143	0.226	0.133	0.091	0.282
A. sojae	6.4	6.0	6.4	0.025	0.027	0.008	0.221	0.096	0.225	0.089	0.089	0.270
A. mellsus	6.2	6.0	6.2	0.077	0.121	0.056	0.181	0.096	0.207	0.193	0.093	0.238
A. ochracius	6.4	6.0	6.6	0.056	0.061	0.020	0.237	0.082	0.269	0.232	0.108	0.294

* pH was measured with respect to the Koji extracts diluted tenfold with water.

To form acid protease from black Aspergillus molds by cultivation in liquid (submerged) according to the invention, various concentrations of inorganic nitrogenous sources e.g. ammonium chloride or sodium nitrate, are added to the above-identified medium containing organic nitrogenous source in a higher concentration. The results in the case of using a black Aspergillus mold, *Aspergillus Saitoi* are shown in the following Table 3.

TABLE 3

Effect of concentration of inorganic nitrogen compounds in the medium to acid protease formation by use of Asp. Saitoi (30° C., 87 hr., 140 r.p.m.)

Addition of Nitrogen Compounds (%)	Ammonium Chloride				Sodium Nitrate			
	C/N	pH	Protease	Yield %	C/N	pH	Protease	Yield %
Control 0	3.15	4.7	0.268	100				
0.25	2.18	4.7	0.324	128	2.41	4.3	0.252	94
0.5	1.60	4.5	0.372	139	1.95	4.5	0.322	120
1.0	1.07	4.5	0.416	155	1.42	4.0	0.380	142
1.5	0.80	4.6	0.424	158	1.17	4.6	0.396	148
2.0	0.64	4.8	0.412	154	0.91	4.3	0.378	141
Defatted Soybean 0.5	2.70	3.8	0.235	87.7				
1.0	2.39	3.6	0.229	85.4				

The C/N ratio in the medium used in the experiment is far lower than that known heretofore (namely the medium containing much more concentrated nitrogen). It is found from the experiment that the maximum yield is obtained when 1% of ammonium chloride or sodium nitrate is added to the basal medium containing a concentrated organic nitrogen source, compared with the control.

When excleated (defatted) soybeans, one of the organic nitrogenous sources, is added to the control, the yield is lower than in the control.

The formation of acid protease in the foregoing basal media (C/N ratio is 3.2) added with each 1% of various kind of organic ammonium salts and nitrates is investigated by shaking cultivation for 63 hours and 87 hours. The results are set forth in Table 4.

TABLE 4

Effect of various kinds of inorganic nitrogen compounds (1%) added to the medium to acid protease formation by use of Asp. Saitoi (30 C., 140 r.p.m.)

Nitrogen comp.	63 hr.				87 hr.		
			Protease			Protease	
	C/N	pH	O.D.	Yield	pH	O.D.	Yield
Control	3.15	3.5	0.150	100	3.9	0.181	100
NH_4Cl	1.07	4.3	0.261	174	4.7	0.334	174
$(NH_4)_2SO_4$	1.22	3.9	0.229	153	4.4	0.275	138
NH_4NO_3	0.87	4.1	0.199	132	4.8	0.328	165
$(NH_4)H_2PO_4$	1.65	3.6	0.181	124	4.1	0.330	166
$(NH_4)_2HPO_4$	1.22	4.0	0.058	39	3.6	0.282	142
$NaNO_3$	1.42				4.1	0.269	149
KNO_3	1.55				4.0	0.239	132
NH_4-citrate	1.64	3.8	0.067	44	3.8	0.244	122
NH_4-tartrate	1.48	4.2	0.018	743	4.2	0.246	124

The experiment shows that addition of each of the ammonium salt increases the formation of acid protease even within a short cultivation period, and that addition of every inorganic nitrogenous source gives 40—80% increased yields after 87 hours cultivation.

Figure 4 is a graph showing variation of the amount of acid protease formed with time, for a 1% addition of an ammonium salt and of a nitrate.

Various types of mold belonging to Kuro-Koji mold group are cultivated in a liquid medium under the afore-said optimum culture condition having a lowered C/N ratio by addition of inorganic nitrogen sources. The results for formation of acid protease are shown in the following Table 5.

TABLE 5

Effect of inorganic nitrogen compounds in the medium for the formation of acid protease by various kinds of black Aspergilli (30° C., 64 hr., 140 r.p.m.)

Strain	Control				1% Ammonium Chloride				1% Sodium Nitrate			
			Protease				Protease				Protease	
	C/N	pH	O.D.	Yield	C/N	pH	O.D.	Yield	C/N	pH	O.D.	Yield
A. Usamii	1.48	4.1	0.271	100%	0.63	5.4	0.286	106%	0.81	4.3	0.382	141%
A. Saitoi	3.15	3.6	0.265	100	1.07	4.0	0.392	148	1.42	3.7	0.329	124
A. aureus	4.70	6.8	0.100	100	1.33	4.5	0.058	58	1.81	6.5	0.115	115
A. awamori	4.70	6.0	0.134	100	1.33	6.2	0.044	33	1.81	5.2	0.210	157
A. niger NRRL—330	2.62	6.6	0.021	100	0.95	7.1	0.010	48	1.29	6.5	0.023	110
A. japonicus	7.58	5.4	0.019	100	1.64	3.5	0.025	132	2.31	6.1	0.016	84

Until now, there have been a number of researches on control of pH in cultivation in liquid of fungi, for example, as to α-amylase of *Aspergillus niger* NRRL—337 (E. H. Le Mense et al, J. Bact. 54, 149 47), as to acid α-amylase of *Aspergillus awamori var. fumeus* (Minoda, J. of Agr. Chem. Soc. of Japan, *35*, 479 and 481 (1961)), as to protease of *Aspergillus oryzae* (M. E. Maxwell, Aust. J. Sci. Res. B5, 42 (1952)), and as to protease of *Aspergillus niger* (G. Gorbach and O. G. Koch, Arch für Mikrobiol. *23*, 265 (1955)).

The inventors have considered the effect of pH on formation of acid protease having an optimum pH at 2.7 by black Aspergillus molds belonging to the Kuro-Koji mold group in a cultivation in liquid, as follows. The present protease having resistivity to acid is stable within a broad pH range of 2.5 to 6.0 when a substrate is present. (See F. Yoshida and M. Nagasawa, Bull. Agr. Chem. Soc. Japan *20*, 257 (1956)). The variation of pH with time in cultivations of *Aspergillus Saitoi* in a medium containing a highly concentrated organic nitrogen source and in the same medium containing also an inorganic nitrogen source are shown in Figure 5. In general, the pH decreases to pH 3.0 to 4.0 after about 40 hours, and, thereafter, gradually increases to about 5.0. In the case where a nitrate is added, however, the decrease in pH tends to be somewhat delayed until 40 hours, and the curve traces the similar way as that in the control. It has been said heretofore that a medium inclines to acid by addition of a physiologically acidic salt, and inclines to alkaline by addition of a physiologically alkaline salt. But no remarkable change is observed in the formation of acid protease in a medium containing highly concentrated nitrogen present in the form of various kind of salts using black Aspergillus type molds, such as *Aspergillus Usamii*, *Aspergillus Saitoi* and the like. In case of *Aspergillus aureus*, *Aspergillus awamori*, *Aspergillus Inuii*, and the like, a rapid increase in pH is observed after 60 hours, and the acid protease found is rapidly decreased with increase of the pH to 6.0 or higher.

Accordingly, the pH in the formation of acid protease using black Aspergillus type molds is to be so controlled that the initial pH is adjusted to about 6.0 which is suitable for germination of black Aspergilli, that the lowest

pH reached after about 40 hours is controlled so as not to be lower than 2.5, and that the cultivation after about 40 hours is carried out within the pH range of 2.5 to 6.0, within which the acid protease of black Aspergilli is stable.

WHAT WE CLAIM IS:—

1. In the production of protease having resistivity to acid and having an optimum pH for milk casein digestion at approximately 2.7 by cultivation of a mold of black Aspergillus type, the improvement comprising adding an inorganic nitrogenous source compound to the cultivation medium.

2. The improvement according to the claim 1, wherein said cultivation is a solid cultivation which is carried out at about 30° C. for at least 60 hours.

3. The improvement according to the claim 1, wherein said cultivation is a cultivation in liquid which is carried out at about 30° C. for at least 60 hours, the pH during said cultivation being kept in the range of 2.5 to 6.0.

4. The improvement according to the claim 3, wherein the mold of black Aspergillus type is selected from the group consisting of *Aspergillus Usamii* and *Aspergillus Saitoi*, and the C/N ratio of the medium is below 3.2.

5. The improvement according to the claim 3, wherein the mold of black Aspergillus type is selected from the group consisting of *Aspergillus aureus* and *Aspergillus awamori*, and the C/N ratio of the medium is approximately 5.

6. The improvement according to the claim 3, wherein the mold of black Aspergillus type is *Aspergillus Inuii* and *Aspergillus Nakazawai*, and the C/N ratio of the medium is approximately 8.

7. The improvement according to the claim 1, wherein said organic nitrogenous source compound is selected from the group consisting of NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, $NaNO_3$ and KNO_3.

8. The improvement according to claim 2, wherein the amount of said inorganic nitrogenous source compound added is 0.5% to 5% based upon the weight of the basal solid medium.

9. The improvement according to claim 3, wherein said inorganic nitrogenous source compound added is an inorganic ammonium salt in amount of 0.25% to 3% based on the weight of the basal solid medium.

10. The improvement according to the claim 3, wherein said inorganic nitrogenous source compound added is an inorganic nitrate in amount of 0.5% to 3% based on the weight of the basal solid medium.

11. A method of producing protease, having high resistivity to acid and having an optimum pH for milk casein digestion at 2.7, substantially as herein described with reference to the drawings.

12. Protease whenever produced by a method according to any of claims 1 to 11.

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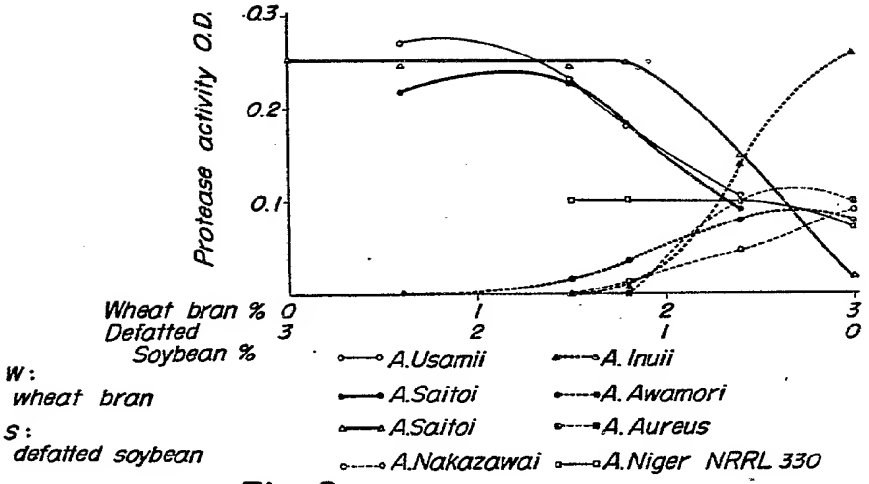
Sheet 1

Fig. 1



W: wheat bran
S: defatted soybean

Fig. 2

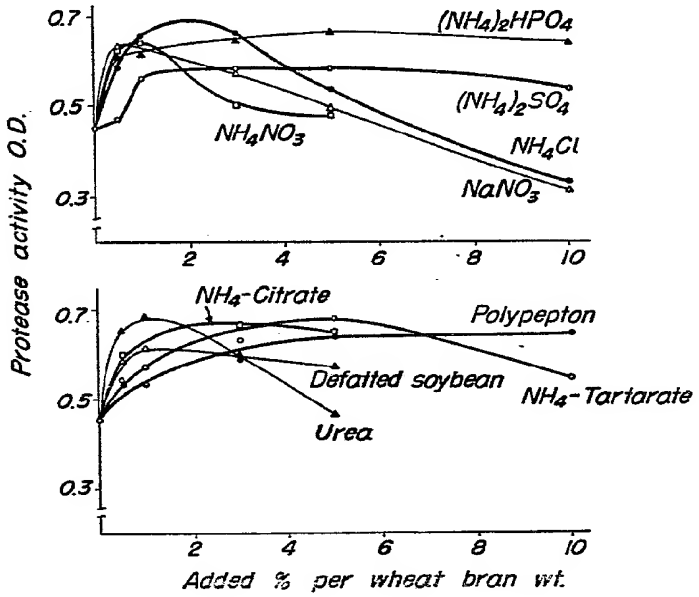


Fig. 3

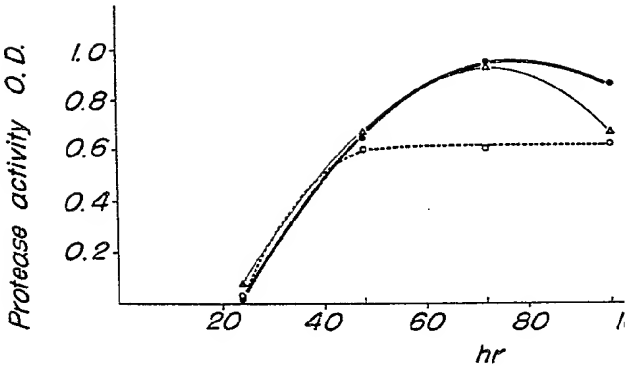
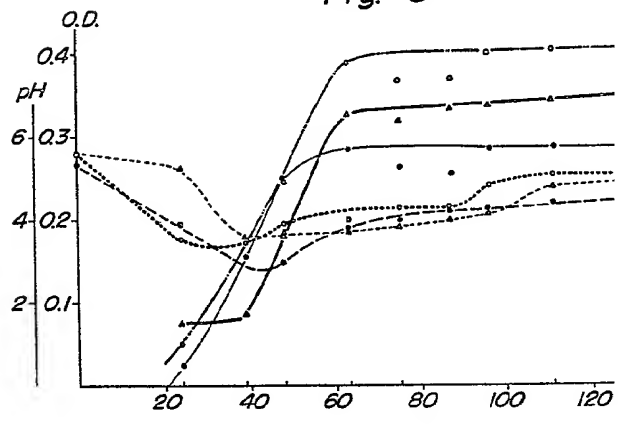


Fig. 5



Wheat bran 1.8%, defatted soybean

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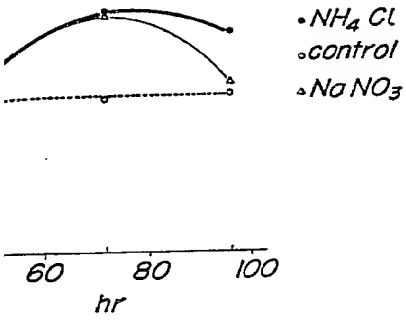

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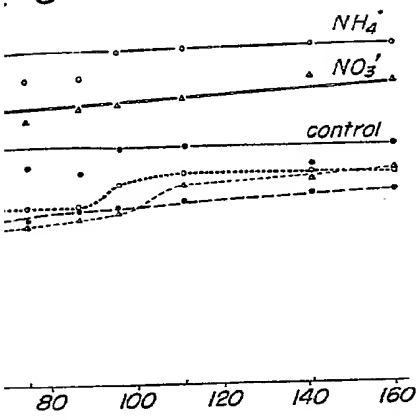

ne (hr)
% , defatted soybean 1.2 %

Fig. 4

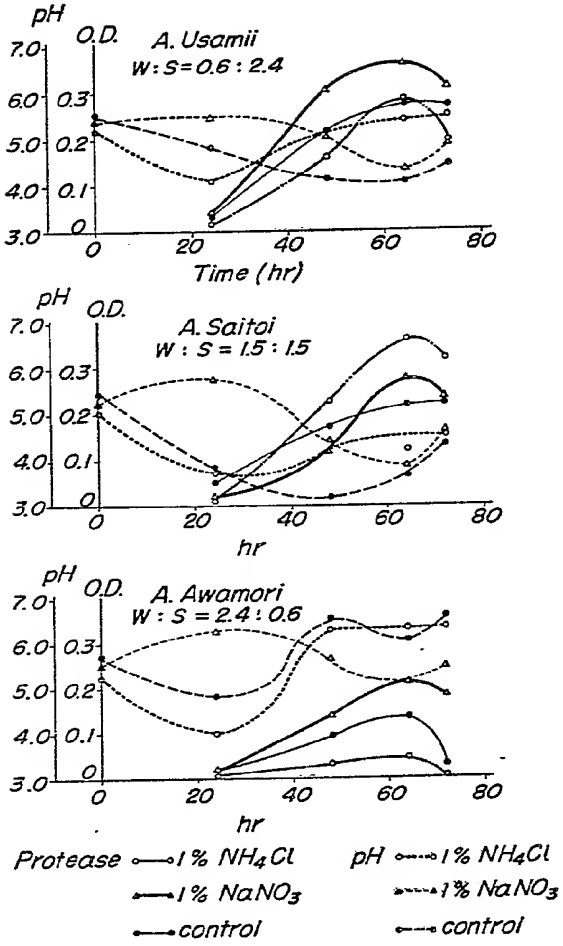

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Fig. 3

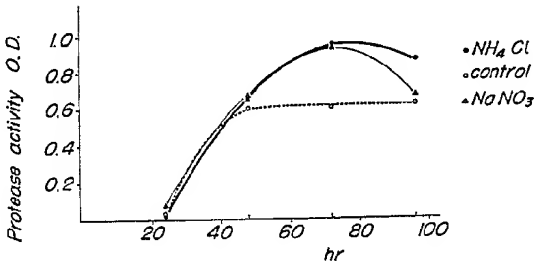

Fig. 4

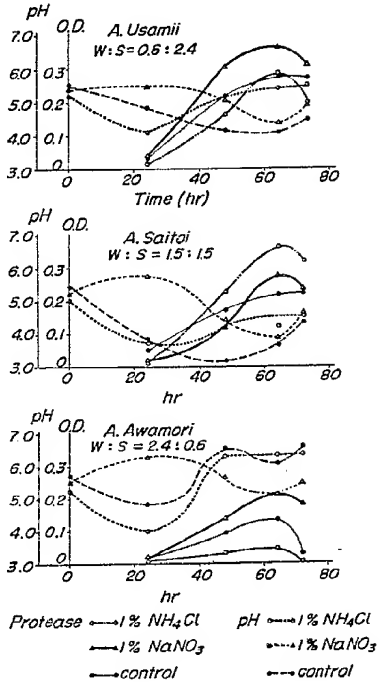

Fig. 5